American Journal of Hematology 54:282–287 (1997)

Brefeldin A-Induced Alterations in Processing of MHC Class II-Ii Complex Depend Upon Microtubular Function

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The role of microtubules in the brefeldin A (BFA)-associated relocation of major histocompatibility complex (MHC) class II αβ chains (αβ) and the invariant chain (Ii) was characterized in Raji cells by the use of nocodazole (ND). BFA blocked the transport of αBIi proteins through the Golgi and redistributed them to the endoplasmic reticulum (ER) along with Golgi-resident enzymes. The result of the colocalization of processing enzymes and newly synthesized proteins was a downshift of αβIi molecular weight (MW) of 2 kDa, and their resistance to endoglycosidase H (endo H) after 6 hr of chase. ND by itself had no effect on the processing and transport of αβ to the cell surface. The addition of ND to BFA-treated cells downshifted αβIi by 4 kDa. Additionally, αβIi proteins remained sensitive to neuraminidase after 16 hr of chase. In vitro α-mannosidase treatment of immunoprecipitated αβIi generated a similar 4-kDa downshift of MW. Either 1-deoxymannojirimycin (DJN) or swainsonine (SWN) blocked the MW downshift caused by BFA + ND treatment. These observations indicated that in Raji cells, most of the BFA-associated relocations of *cis*-, medial Golgi proteins, and the addition of sialic acid from the *trans*-Golgi were microtubule-independent. The retrograde transport of the medial Golgi enzyme N-acetylglucosamine transferase, however, required microtubular function. Microtubule disrupters could affect BFA treatment of viral infections by further disrupting viral protein processing. Am. J. Hematol. 54: 282–287, 1997. © 1997 Wiley-Liss, Inc.

Key words: MHC class II-Ii; brefeldin A; nocodazole

INTRODUCTION

Brefeldin A, a fungal antibiotic, blocks the transport of proteins from the endoplasmic reticulum (ER) [1], and redistributes to the ER the itinerant and resident proteins from *cis*-, medial, and *trans*-Golgi by a retrograde transport mechanism which depends on microtubule functions [2–4]. Processing events in the *trans*-Golgi network, however, are not affected by brefeldin A (BFA) [5]. BFA inhibits both major histocompatibility complex (MHC) class II- and class I-restricted antigen presentation [6,7] and the maturation and egress of several viruses [8–11]. The addition of nocodazole, a reversible blocker of tubule polymerization [12], to BFA-treated cells, was associated with the inhibition of such recycling transport in normal rat kidney cells [3].

To characterize the impact of BFA and of the combination of BFA plus nocodazole (ND) on antigen presentation and lymphocyte maturation, we elected to define the alterations of the processing of MHC class II-Ii complexes in Raji cells. These changes define the requirement for microtubular function in the BFA-associated redistribution of Golgi enzymes. MHC class II αβ chains associate with Ii in the ER after synthesis. αβIi complexes are transported through the Golgi and processed by Golgi enzymes. Processing leads to the dissociation of Ii from the αβIi complex to catalyze the charging of antigenic peptide to MHC class II [13–17]. We found

Abbreviations: BFA, brefeldin A; MHC class II, class II major histocompatibility complex; DJN, 1-deoxymannojirimicin; endo H, endoglycosidase H; ER, endoplasmic reticulum; Iad, immunoabsorbent; Ii, invariant chain; mAb, monoclonal antibody; ND, nocodazole; SWN, swainsonine.

Contract grant sponsor: American Heart Association; Contract grant numbers 900868 and 900441; Contract grant sponsor: Children's Miracle Network; Contract grant number: 916.

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Received for publication 31 May 1996; accepted 6 November 1996.

that in BFA-treated Raji cells, ND did not block the retrograde distribution of measured Golgi proteins, except for N-acetylglucosamine transferase. Such blockage could affect the therapeutic potential of BFA or BFA-like drugs in viral diseases.

MATERIALS AND METHODS

Cells and Antibodies

Raji lymphoblastoid cells were tested to be free of mycoplasma and maintained in RPMI 1640 (GIBCO, Grand Island, NY) containing 10% fetal calf serum. 9.3F10, which recognized mature human MHC class II, was a monoclonal antibody (mAb) raised from HB-180 (ATCC, Rockville, MD). VIC-Y1, an IgG_1 mAb to human Ii, which recognized Ii and associated MHC class II, was obtained from An Der Grub (Kaumberg, Austria) [18].

BFA and ND Treatment

BFA, a gift from Sandoz (Basel, Switzerland), was made into a stock solution at 5 mg/ml in dimethyl sulfoxide (DMSO), and used at 2.5 μg/ml to treat cells from the beginning of metabolic radiolabeling. ND (Sigma, St. Louis, MO) was made into a stock of 16.5 mM in DMSO and diluted to a final concentration of 3.3 μM in culture medium. ND treatment preceded other manipulations such as metabolic labeling or BFA treatment by 2 hr.

Glycosidase Digestions

Immunoprecipitated proteins were denatured by 0.5% SDS in 0.15 M Na acetate buffer, pH 4.75, and were boiled for 2 min before overnight incubation with α-mannosidase (Boeringher Mannheim, Indianapolis, IN) in 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5% Triton X-100. Samples were boiled for 2 min to stop enzyme activity.

Endoglycosidase H (Endo H) and neuraminidase treatments of [^{35}S] methionine-labeled, immunoprecipitated proteins from 5×10^6 cells were performed as described elsewhere [19]. Briefly, after an overnight incubation with endo H (5 microunits in 0.15 M Na citrate, pH 5.5, 1 mM PMSF) and neuraminidase (100 milliunits in 0.1 M Na acetate, pH 5.5, 0.3 M NaCl and 0.2% $CaCl_2$, 1 mM PMSF), proteins were electrophoresed by SDS-PAGE, followed by autoradiography.

Mannosidase Inhibitors

1-deoxymannojirimycin (DJN, Genzyme, Boston, MA) and swainsonine (SWN, Boehringer Mannheim) were made into stock solutions in DMSO and serially diluted in culture media at final concentrations of 1 mM DJN and 2 μg/ml SWN. Cells were incubated for 1 hr with inhibitors prior to other treatments.

Radiolabeling and Immunoprecipitation

Ten million Raji cells were incubated for 30 min in methionine-free RPMI 1640 prior to a 15-min pulse radiolabeling with 1 mCi [^{35}S] methionine. Cells were washed by centrifugation in PBS containing 4 mM L-methionine, and incubated with medium lacking methionine during chase times.

For immunoprecipitation of cell lysate proteins, pellets from 5×10^6 metabolically labeled cells were lysed in 1 ml of lysing buffer (100 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5% w/v Triton X-100, 0.02% NaN_3, 2 mM PMSF, and 10 mM N-ethylmaleimide). The lysate was centrifuged at 35,000 rpm at 4°C for 30 min, and the supernatant was collected. A specific immunoadsorbent (Iad) was prepared by incubating 100 μl of preswollen protein A-sepharose (Sigma) with 2 μl of a mAb on a rocker at 4°C for 4 hr. A washed Iad was incubated with cell lysate at 4°C for 16 hr.

For immunoprecipitation of cell surface proteins, after [^{35}S] methionine metabolic labeling and chase times, 5×10^6 cells were incubated with 2–10 μl of a specific mAb at 4°C for 8 hr. Cells were lysed in the presence of a 10-fold excess of unlabeled cells, and 100 μl of protein A-sepharose were added to form an Iad.

Immunoprecipitated proteins were eluted from Iad with Laemmli sample buffer or IEF sample buffer. Proteins were separated by 10% SDS-polyacrylamide gels (SDS-PAGE), followed by autoradiography. MW of protein bands was calculated using panels of protein MW standards whose migration was scanned and analyzed by a Biorad GS 670 densitometer with the Molecular Analyst™ Macintosh version 2.1 program Biorad, Hercules CA.

RESULTS

Alterations in Processing of MHC Class II-Ii in Cells Incubated With BFA, ND, or BFA + ND

To compare the differences in MHC class II and Ii processing associated with each drug or a combination of both, Raji cells underwent a 15-min pulse label with radiomethionine during drug treatment. Cell aliquots were chased for 0, 2, 6, and 16 hr without drug renewal. After 16 hr chase, protein bands revealed marked differences. In untreated cells, most of the VIC-Y1-immunoprecipitated proteins disappeared by 16 hr. BFA treatment was associated with persistence of Ii at 16 hr, although without p41 (an alternately translated Ii form with an extra exon encoding for 64 amino acids). Densitometric measurements of protein bands revealed a progressive downshift in MW of 2 kDa which affected equally all species (α, β, Ii, and p41). The BFA + ND treatment lowered the Ii main band 4 kDa to 31 kDa after 16 hr of chase (Fig. 1).

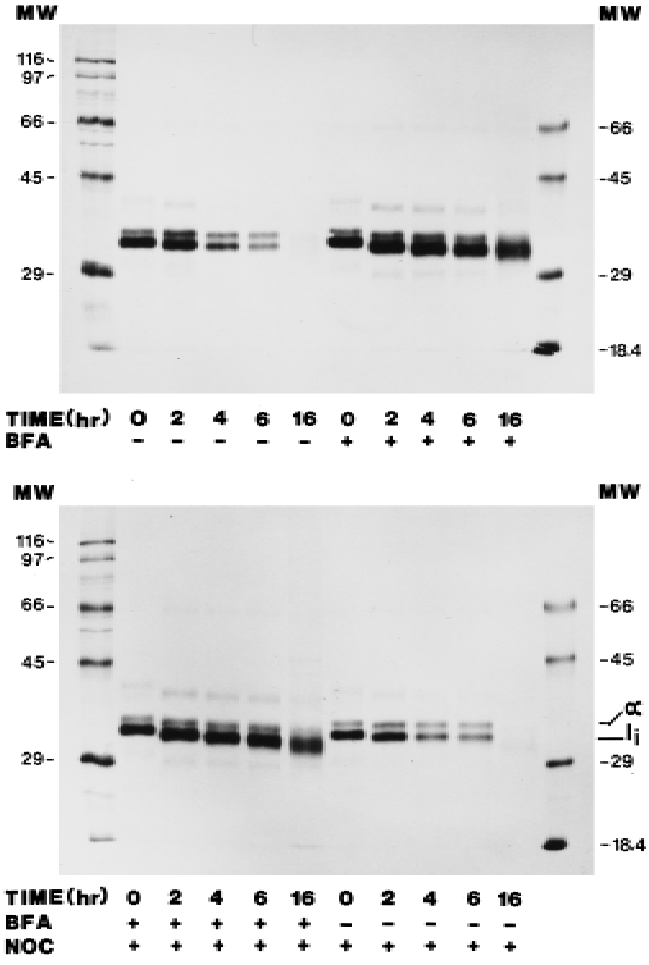

Fig. 1. Changing pattern of processing of αβIi in cells treated with brefeldin A, nocodazole, or both after 16 hr of chase. VIC-Y1 immunoprecipitations of radiomethionine-labeled Raji cell lysates showed that brefeldin A-treated samples were associated with the persistence of Ii, and a progressive 2 kDa downshift of the main bands' molecular weight (MW) after 16 hr of chase (top). Such a lowering of MW was up to 4 kDa in samples treated with both brefeldin A and nocodazole. Nocodazole-treated samples showed no changes as compared to controls (bottom). BFA, brefeldin A; ND, nocodazole; α, MHC class II α chains; Ii, invariant chain.

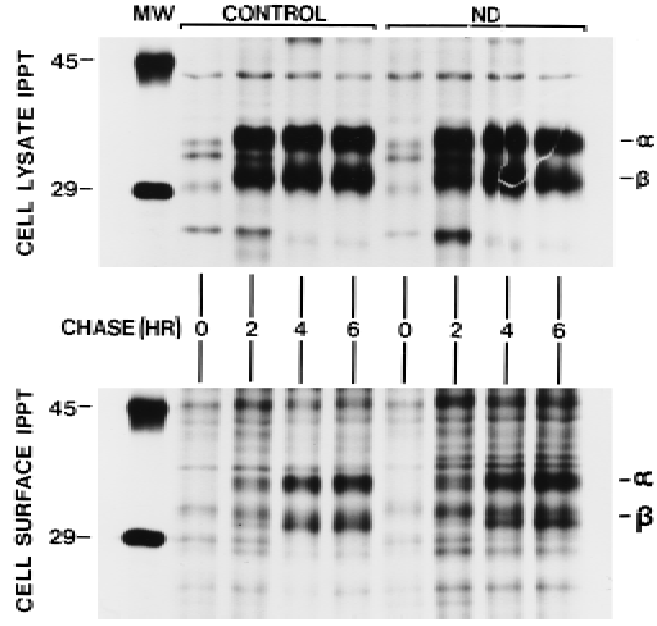

Fig. 2. Nocodazole treatment alone did not change the processing nor the transport to the cell surface of MHC class II molecules. Raji cells treated with nocodazole did not show any changes in the processing of αBIi (top), or in the level of surface expression of MHC class II at indicated chase times (bottom). ND, nocodazole.

In contrast, ND did not alter the synthesis and processing of either MHC class II or Ii (Fig. 1, and Fig. 2, top), and had no effect on the transport of MHC class II to the cell surface (Fig. 2, bottom).

Class II-Ii Proteins from BFA + ND-Treated Cells Were Resistant to Endo H But Sensitive to Neuraminidase

To document major processing events by Golgi enzymes, we used endo H and neuraminidase to treat αβIi proteins immunoprecipitated by VIC-Y1 from cells incubated with BFA + ND. By 6 hr of chase, αβIi became resistant to endo H (Fig. 3B), which was similar to BFA treatment alone [20]. αβIi proteins became sensitive to neuraminidase after 2 hr of chase and till the end of 16 hr of chase (Fig. 3C).

Downshift of MW Was Due to Action of Golgi Mannosidase

We next tested whether the downshift of αβIi proteins from Raji cells was due to the action of glycosidase-trimming enzymes. VIC-Y1-immunoprecipitated proteins were incubated with α-mannosidase for 16 hr, which downshifted the MW by approximately 4 kDa (Fig. 4). This finding was similar to the effect of 16 hr of treatment of cells with BFA + ND (Fig. 1).

1-deoxymannojirimicin and Swainsonine Blocked Downshift of MW Caused by BFA + ND

To investigate the reason for the MW downshift of αβIi proteins, we used DJN and SWN to treat cells during their incubation with BFA + ND. DJN blocks the action of Golgi mannosidase I, while SWN inhibits Golgi mannosidase II. Neither of these drugs caused any decrease in MW of αβIi proteins [21]. The addition of DJN or SWN at the beginning of BFA + ND treatment, however, blocked the downshift of MW of the protein bands representing Ii (Fig. 5, top) and αβ (Fig. 5, bottom).

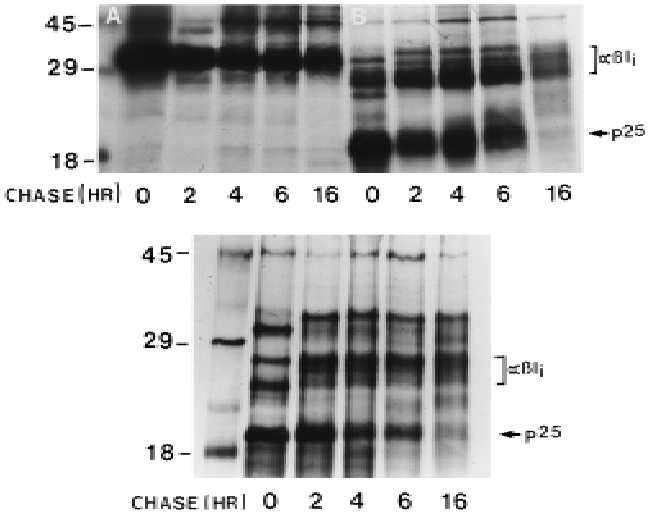

Fig. 3. αβIi proteins from cells treated with a combination of brefeldin A plus nocodazole were resistant to endo H after 6 hr (B), but remained sensitive to neuraminidase (bottom) from 2–16 hr of chase. A: Untreated control. The abundance of p25, a digested C-terminal product of Ii, was probably due to a denaturing condition used in enzyme digestions (B, bottom).

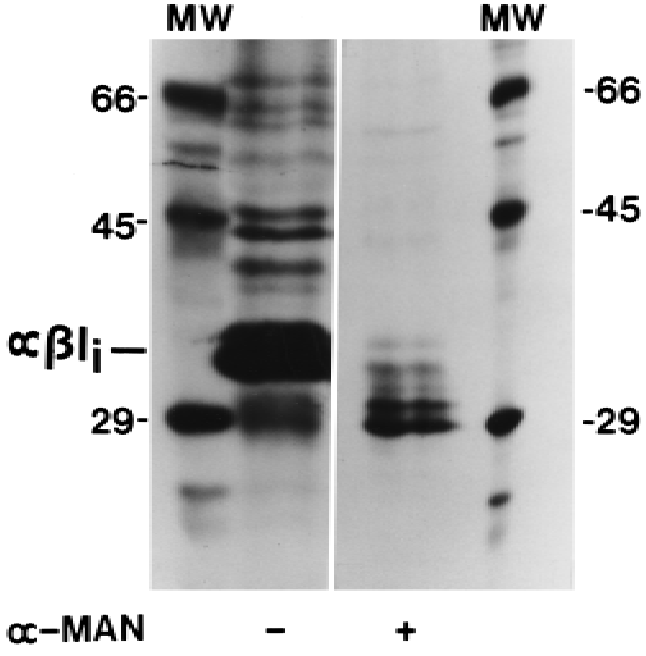

Fig. 4. In vitro α-mannosidase treatment of pulsed-chased cell lysates after immunoprecipitations by VIC-Y1. The main band of Ii was reduced to about 31 kDa after overnight treatment with α-mannosidase. MW, molecular weight in kDa; α-MAN, α-mannosidase.

DISCUSSION

We characterized the altered processing of MHC class II-Ii proteins due to their colocalization with redistributed Golgi glycosidases in Raji cells treated with BFA, ND, and a combination of both drugs to define the microtubular requirement of BFA-induced redistribution in human cells.

Newly synthesized proteins emerge from the ER as N-linked oligosaccharides, which are trimmed from high-mannose forms to $Man_5GlcNAc_2$ by α-1, 2-mannosidase I in the *cis*-Golgi. The *cis*-Golgi enzyme, N-acetylglucosamine transferase I, quickly adds N-acetylglucosamine (GlcNAc) to $Man_5GlcNAc_2$ oligosaccharides. Golgi-mannosidase II cleaves two mannose molecules, so that GlcNAc can be added to yield GlcNAc-Man_3-$GlcNAc_2$ complex saccharides, which become endo H-resistant after the addition of one more GlcNAc [22]. Finally, additional GlcNAc, galactose, and sialic acid residues are added in the *trans*-Golgi.

BFA inhibits all Golgi processing events, except the steps in the *trans*-Golgi network [5,23], by causing a retrograde transport of Golgi resident and itinerant proteins to the ER. Even then, the role of the retrograde transport of proteins to the ER is not well-understood. It is believed that it occurs in vivo, probably to retrieve ''escaped'' ER proteins and control the rate of transport through the Golgi [3].

However, the effect of BFA may be dependent on cell type. For example, the addition of sialic acid, a *trans*-Golgi network function, occurred in BFA-treated Raji cells (Fig. 3C) but was not observed in BFA-treated Chinese hamster ovary (CHO) cells [4,23]. Furthermore, Sreenivasan et al. reported that BFA inhibited the replication of West Nile virus in Vero cells, but not in C6/36 (a [24] mosquito cell). In Raji cells, BFA treatment downshifted the MW of MHC class II-Ii proteins and rendered them resistant to endo H, which suggested their encounter with medial Golgi processing enzymes [20]. Similar processing of G proteins was shown in CHO 15B cells, which were deficient in N-acetylglucosamine transferase [4].

The requirement of microtubule function for the BFA-induced retrograde distribution of Golgi proteins was documented in normal rat kidney fibroblast (NRK) cells [3]. In Raji cells, the addition of ND to BFA-treated cells was associated with a 4-kDa downshift of αβIi. The downshift of MW of processed αBIi generated by α-mannosidase in vitro treatment showed that the trimming by Golgi mannosidase was enough to cause the 4-kDa downshift of MW. In the absence of N-acetylglucosamine transferase, Golgi itinerant proteins were only exposed to Golgi mannosidases, and as a result, were digested of five mannose residues, which manifested themselves as downshift in MW. These results suggested that the 4-kDa downshift of αβIi proteins associated with the treatment with a combination of BFA and ND for 16 hr could be interpreted as the accumula-

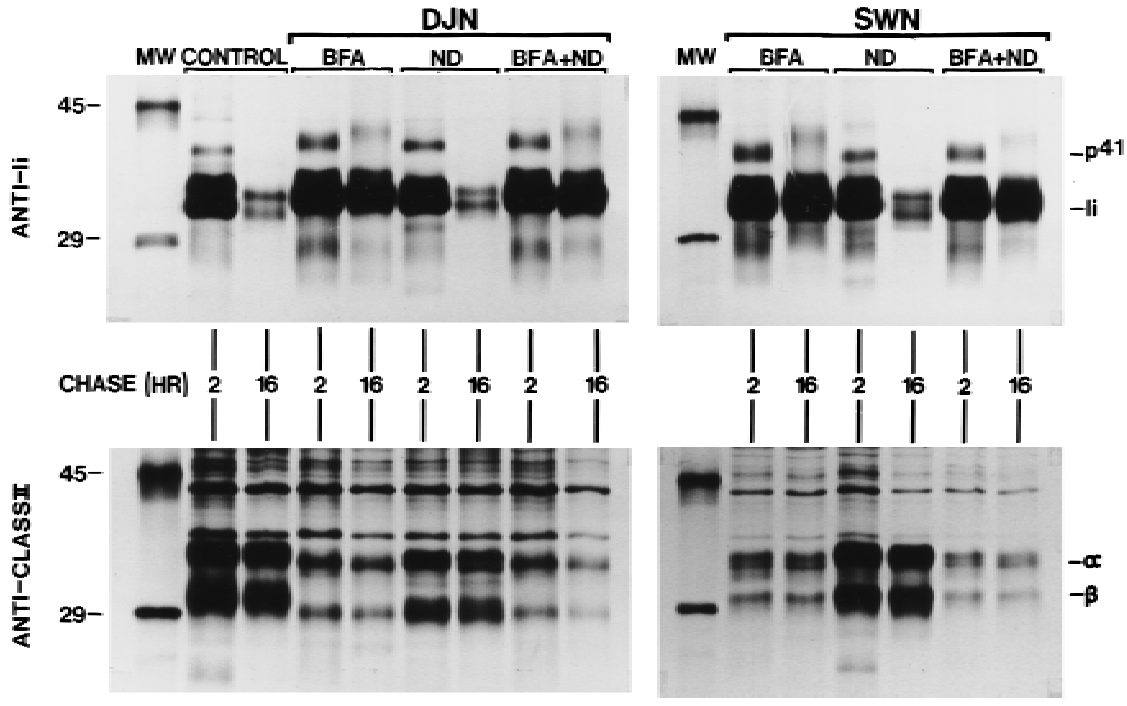

Fig. 5. Incubations with either deoxymannojirimycin or swainsonine blocked the MW downshift of the combined brefeldin A plus nocodazole treatment of cells. The presence of deoxymannojirimycin or swainsonine was also associated with the increased presence of αβ mature chains in BFA-treated samples. Control, sample treated with deoxymannojirimycin (there was no change in sample treated with swainsonine; results not shown); DJN, deoxymannojirimycin; SWN, swainsonine.

tive results of the lack of the addition of GlcNAc in the presence of the continuous action of mannosidase. Our observations suggest that the microtubular requirements for retrograde distribution of Golgi resident and itinerant proteins are different among mammalian cells. Specifically, in human Raji lymphoblastoid cell lines, most of the BFA-associated relocations were not inhibited by ND.

The blockers of glycosidase-trimming enzymes such as 1-deoxymannojirimicin and swainsonine abolished the downshift associated with the combined treatment of BFA and ND on αβIi proteins. The inhibition of either Golgi mannosidase I (by DJN) or Golgi mannosidase II (by SWN), i.e., each of the glycosidases which precede a Golgi N-acetyl glucosamine transferase, arrested the processing of Golgi itinerant proteins at Man_8-$GlcNAc_2$-Asn and GlcNAc-Man_5-$GlcNAc_2$-Asn forms, respectively. This arrest of processing probably inhibited further action by other Golgi enzymes and consequently blocked any downshift of the MW of newly synthesized proteins.

BFA, which inhibited protein processing and assembly of several viruses infectious to humans [8–11], could potentially be used as a therapeutic agent. ND and similar compounds, which have been used in Grave's disease in humans [25] and in the prevention of systemic lupus erythematosus in animal models [26], could interfere with inhibitory effects of BFA on viral infection of mammalian cells. Interactions between these two reagents on the synthesis and assembly of various viral pathogens remain to be elucidated in human cells.

ACKNOWLEDGMENTS

We thank Drs. R.E. Humphreys and R.C. Spiro for their critical reviews, and Ms. M. Hogan for the preparation of this manuscript. This study was supported in part by grants 900868 and 900441 from the American Heart Association, and by grant 916 from the Children's Miracle Network.

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